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## Annual Report

**Title of the Grant Proposal:** Exploration of the regulation of breast cancer by the Angiotensin II receptor AT2

**PI: Lakshmi Pulakat**

### Abstract:

The Angiotensin II (Ang II) receptor subtype AT2 is a protein with seven transmembrane domains. It was shown that the third Intracellular Loop (3<sup>rd</sup> ICL) of this receptor is needed for the induction of apoptosis in some cell lines. Our recent studies suggest that the 3<sup>rd</sup> ICL of the AT2 is also involved in reducing cGMP levels in oocytes and this effect is mediated through sequestering Gi alpha and possible activation of Gi beta-gamma subunits. Thus the 3<sup>rd</sup> ICL of the AT2 plays a crucial role in the functions of this receptor. To further characterize signaling by the AT2, we had used yeast two-hybrid assay to identify the proteins that may directly interact with the AT2. These studies lead to the identification of a direct protein-protein interaction between the ATP binding domain of the ErbB2 and ErbB3 receptors and the AT2. A chimeric receptor in which the 3<sup>rd</sup> ICL of the AT2 was replaced with that of the AT1, and a truncated AT2 receptor that did not have the C-terminal cytoplasmic domain were unable to interact with the ATP binding domain of the human ErbB3 receptor. Moreover deleting the C-terminus of the AT2 also abolished the interaction between the AT2 and the ErbB3. Therefore the 3<sup>rd</sup> ICL and C-terminal cytoplasmic domain of the AT2 are essential for this interaction. The receptors of the ErbB family, particularly ErbB2 and ErbB3 are overexpressed and constitutively phosphorylated in many breast cancer cells and such overexpression suggests poor prognosis. Since the 3<sup>rd</sup> ICL of the AT2 is involved in growth regulation and this region is needed for the interaction between the AT2 and the human ErbB3, we hypothesized that this interaction may result in growth regulation of the breast cancer cells that overexpress ErbB2 and ErbB3. Recently it was shown that AT2 forms heterodimers with the Angiotensin II receptor AT1 and inhibits the AT1-mediated IP<sub>3</sub> production. This observation further supports our hypothesis that direct interaction between the AT2 and another membrane bound receptor such as ErbB2 or ErbB3 may result in inhibition/regulation of growth-promoting signaling by these receptors. To test this hypothesis we used the breast cancer cell line MDA-MB-453. Our ligand-binding experiments and RT-PCR studies showed that this cell line does not have any AT2 receptor. Moreover, Immunoprecipitation and Western-blotting studies showed that this cell line has high-level expression and constitutive phosphorylation of both ErbB2 and ErbB3 receptors. Therefore we have started generating derivatives of MDA-MB 453 cells carrying the wild type AT2 by introducing the wildtype AT2 into these cells on the mammalian vector pCDNA. We observed that our attempts to generate stable cell lines that express the wildtype AT2 were not very successful since the cells that showed resistance to G418 (suggesting that they contained pCDNA derivative carrying the wildtype AT2) showed highly reduced growth. This was not surprising since the AT2 is known to have the ability to inhibit cell growth. Therefore we have generated mammalian vectors expressing the AT2 and a mutant AT2 (in which the third intracellular loop of the AT2 is replaced with that of the AT1) by cloning these genes in pRevTRE (for regulated expression of the gene by tetracycline). Currently we are in the

process of generating MDA-MB-derivatives expressing the AT2 and the chimeric receptor using the pRevTRE constructs. Our next step is to analyze how the AT2 expression is affecting the high-level expression and constitutive phosphorylation of the ErbB2 and the ErbB3 receptors in the MDA-MB 453 cells. We have been granted a One-year no-cost extension to complete these experiments.

## **Introduction**

The aim of this research proposal is to investigate if the overexpression and activation of the angiotensin II receptor AT2 causes inhibition of cell growth and promotion of cell differentiation in breast cancer cells that overexpress the ErbB2 and ErbB3 receptors. The ErbB or subclass 1 receptor tyrosine kinase proteins are consisted of four homologous members; the EGFR or ErbB1, ErbB2, ErbB3 and ErbB4. Binding of a number of structurally similar growth factors to the extracellular domain of these receptors induce signaling by these receptors. In response to ligand-binding, the ErbB family receptors may form homodimers or heterodimers. This results in the activation of intracellular kinase domain leading to autophosphorylation of tyrosine residues. Subsequently, the growth signal is propagated through docking of the SH2 and PTB-domain containing proteins to the phosphorylated tyrosine residues and induction of cell-division through mitogen-activated protein kinase and/or S6 kinase pathways (1). The involvement of the ErbB receptor family in the control of cell proliferation and hence its role in human malignancies has led to intense studies of these receptors and their cognate ligands. For example, overexpression of ErbB2 and ErbB3 receptors are correlated to the transformation of breast, ovarian and many other cell types into malignant tumors (2). Identification of a mechanism to regulate growth promoting cell signaling by these receptors may lead to the development of effective treatments to inhibit tumor growth in breast and ovarian cancer types that show ErbB2 and ErbB3 receptor overexpression. The Angiotensin II (Ang II) receptor subtype AT2 is expressed in high levels during fetal development and also after injury and during wound-healing in adults (3). This receptor is shown to exert inhibitory effects on cell growth in many cell types. Moreover, the AT2 is also known to activate differentiation in different cell types. Thus, the AT2 seems to function as a regulator of cell proliferation and activator of differentiation. . Our recent studies show that the region of the AT2 spanning the 3rd intracellular loop (ICL) and the c-terminal can directly interact with the ATP-binding domain of the ErbB2/ErbB3 receptors in a yeast Two-Hybrid protein-protein interaction assay (4). Studies using mutated and chimeric AT2 receptors showed that replacing the 3rdICL of the AT2 receptor with that of the AT1 abolishes the interaction between the ErbB3 and the AT2. Thus the interaction between the AT2 and the ErbB3 involves the 3rd ICL of the AT2. Since the 3rd ICL of the AT2 is essential for exerting its inhibitory effects on cell growth, its involvement in the interaction with the ATP-binding domain of the ErbB3 suggests a novel signaling mechanism for the AT2 receptor-mediated inhibition of cell growth and activation of differentiation of cells. Moreover, it opens up the possibility of using the AT2 receptor peptides in regulating the ErbB2/ErbB3 mediated signaling in breast cancer.

This research stems from the above observation that the growth-inhibiting AT2 interacts directly with the cancer-promoting ErbB2/ErbB3 receptors and involves a number of breast cancer cell lines that overexpress the ErbB2/ErbB3 receptors. Since the domains of the ErbB2/ErbB3 receptors that interact with the AT2 receptor are located within the 21 amino acids that separate the residues GlyXGlyXXGly, and Lys (the residues predicted to be important for ATP binding), it is reasonable to assume that an interaction between the AT2 receptor and the ErbB2/ErbB3 receptors may result in influencing the ATP binding properties of the ErbB2/ErbB3 receptors. This situation may affect the tyrosine kinase activity of the ErbB2/ErbB3 receptors and the ErbB2/ErbB3 receptor-mediated cell proliferation. Moreover, since the AT2 receptor is known to inhibit cell proliferation and promote differentiation in many different cell types, the interaction between the ATP binding domain of the ErbB2/ErbB3 receptors and the 3rdICL and the c-terminal of the AT2 may also cause differentiation of these cancer cells. Thus, the research proposed in this grant proposal is directed to initiate experiments to test this possibility. It was shown that tumor cell lines MDA-MB-453, MDA-MB-175, and SUM-52PE have high level expression and phosphorylation of ErbB2 and ErbB3 receptors. We have been testing these cell lines to see whether they express the AT2 receptor. The experiments proposed in the original proposal were to test

- a) growth arrest
- b) induction of apoptosis,
- c) induction of differentiation,
- d) reduction of phosphorylation of the ErbB2/ErbB3 receptors and
- e) reduction in the overexpression of the ErbB2/ErbB3 receptors.

#### **Key Research Accomplishments:**

1. RT-PCR analysis using appropriate primers corresponding to the 5' and 3' regions of the Angiotensin II receptors AT1 and AT2 and total RNA isoalted form the the breast cancer cell line MDA-MB-453 did not result in the amplification of the DNA fragments corresponding to the human AT1 or the AT2.
2. Ligand-binding experiments using <sup>125</sup>-I labeled Ang II showed no binding to MDA-MB-453 cells indicating that these cells did not express any Angiotensin II receptors.
3. These two results lead us to conclude that the breast cancer cell line MDA-MB-453 did not express either the AT1 or the AT2 receptors of Angiotensin II.
4. The rat AT2 receptor cloned in pCDNA was then introduced into the MDA-MB-453 cell line by stable transfection to generate MDA-MB-453 derivatives expressing the AT2 and the ErbB2/B3 receptors.
5. To do this a pCDNA 3.1 derivative that carry the DNA specifying the open reading frame of the AT2 was made. Nucleotide sequencing was carried out to determine that the orientation of the gene is same as that of the strong CMV promoter that regulates the expression of the AT2 in this construct. The primers used to amplify the DNA specifying the open reading frame of the AT2 also carried the Kozak sequence upstream to the translation initiation codon ATG to ensure that the final construct had all the appropriate signals for efficient translation.

6. The stable transfectants showed highly reduced growth indicating that the AT2 overexpression was causing inhibition of cell growth.
7. This result suggested that as we have anticipated, the constitutive overexpression of the AT2 could inhibit cell growth of this breast cancer cell line.
8. To confirm that this growth inhibitory effect is due to the 3<sup>rd</sup> ICL-mediated signaling of the AT2 we also constructed a pCDNA derivative carrying a chimeric receptor in which the third ICL of the AT2 was replaced with that of the AT1.
9. Currently experiments are in progress to test whether this chimeric receptor also inhibits cell growth of the breast cancer cell line MDA-MB-453 by introducing this chimeric receptor into MDA-MB-453 by transfection.
10. Since the AT2 receptor is expressed constitutively from the pCDNA construct, we decided to create a construct that could express the AT2 in mammalian cells in a regulated manner. For this purpose we used the "Tet-On gene expression system" from ClonTech Inc. The "Tet-On system has two components. First is the Tet-On plasmid that produces the rtTA, the regulatory protein that binds the Tet Responsive Element (TRE) and activates transcription. The second plasmid is pTRE which carries the TRE that contains seven direct repeats of a 42 bp sequence containing the *tetO* upstream of the minimal CMV promoter. We have now cloned the AT2 gene and two AT2 mutants under the transcriptional control of TRE in pTRE vector. These constructs also have Kozak sequence upstream to the translation initiation codon ATG to ensure efficient translation of the AT2 or its mutants. Currently experiments are in progress to introduce these constructs into the MDA-MB-453 and generate cell lines in which the AT2 expression can be turned on only in response to tetracycline.
11. We analyzed the AT2 expression in another breast cancer cell line SKBR3 by SDS-PAGE analysis of the cell lysate, Western blotting and probing with Anti-AT2 antibody. Low level expression of the AT2 was visible in this cell line.

### **Reportable Outcomes**

1. Structure-Function of the Angiotensin II Receptor AT2 and Signaling in Breast Cancer (Abstract submitted on April 1<sup>st</sup>, to 'Era of Hope' Meeting to be held at Orlando, Fl., on Sept 25-29)
2. Dieter Knowle, a Ph.D. student who characterized the interaction between the rat AT2 and the human ErbB3 received his Ph.D. degree on August 2001. His Dissertation was chosen as the "Distinguished Dissertation of 2001" by Bowling Green State University in November 2001.
3. PI (Dr. Pulakat) was awarded "Certificate of Appreciation" by the Distinguished Dissertation Award Committee of Bowling Green State University for her guidance of Distinguished Dissertation Award Recipient, Dr. Dieter Knowle, November 2001.

### **Conclusions**

In summary, to date we have successfully completed construction of the recombinant vectors that can express the AT2 or its mutants either constitutively or under tetracycline-mediated regulation in breast cancer cell lines. We have shown that the overexpression of the AT2 may cause inhibition of cell growth of the breast cancer cell

line MDA-MB-453. This result is very encouraging, since the main goal of this proposal is to test whether the AT2 could inhibit growth of breast cancer cell lines and induce differentiation. We also found that the breast cancer cell SK-BR3 shows low level expression of the AT2 in our experimental conditions. We submitted an abstract describing these results to the 'Era of Hope meeting to be held at Orlando, Fl., in September 2002. Dieter Knowle, a Ph.D. student in the PI's lab who characterized the interaction between the AT2 and the human ErbB3 received his Ph.D. degree in August 2001 and is currently working as a Post-Doctoral Fellow at Medical College of Ohio, Toledo. His dissertation received the "Distinguished Dissertation Award" for the year 2001 by Bowling Green State University and the PI received the "Certificate of Appreciation" by the Distinguished Dissertation Award Committee of Bowling Green State University for her guidance of Distinguished Dissertation Award Recipient, Dr. Dieter Knowle, November 2001. Our experiments are now directed to determine whether the overexpression of the AT2 affects the overexpression of the ErbB2/B3 receptors and phosphorylation of these receptors. Since the stable transfectants of the MDA-MB-453 carrying the AT2 showed highly reduced growth, we needed to use other strategies to generate cell lines in which the effect of AT2 expression could be analyzed carefully. We are confident that one of the two different methods to express the AT2 (either constitutive expression from CMV promoter or regulated expression from promoter with TRE) should result in successful and detectable overexpression of this receptor. We are also using transient transfection to test the effect of the AT2 on the ErbB2/B3 receptor functions in MDA-MB-453. We anticipate we will be able to complete these experiments by April 2003. This is why we had requested for a 12-month no-cost extension for this grant-period.

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